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REPORT NUMBER 4

Pathogenesis of Salmonellosis: Salmonella Exotoxins

Annual and Final Report

Johnny W. Peterson, Ph.D.

March 8, 1982

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

D//,//Di7-77-C-7c54

Contract No. ~~DAM D17 77 C 7054~~

University of Texas Medical Branch
Galveston, Texas 77550

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REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4	AD-A139 007	

4. TITLE (and Subtitle)	5. TYPE OF REPORT & PERIOD COVERED
Pathogenesis of Salmonellosis: Salmonella Exotoxins	Annual (Aug 80–Dec 81) Final (Dec 77 – Dec 81)
	6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)	8. CONTRACT OR GRANT NUMBER(s)
Johnny W. Peterson, Ph.D.	DAMD17-77-C-7154 ~~DAMD17-77-C-7054~~

9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
University of Texas Medical Branch Galveston, Texas 77550	62770A.3M162770A871.AE.053

11. CONTROLLING OFFICE NAME AND ADDRESS	12. REPORT DATE
US Army Medical Research and Development Command Fort Detrick Frederick, Maryland 21701	3/8/82
	13. NUMBER OF PAGES
	21

14. MONITORING AGENCY NAME & ADDRESS(If different from Controlling Office)	15. SECURITY CLASS. (of this report)
	Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, If different from Report)

Approved for public release; distribution unlimited

18. SUPPLEMENTARY NOTES

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MAR 1 2 1984

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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

We are now using several rapid, in vitro methods to quantitate Salmonella toxin contained in culture filtrates and sonicated cell preparations. Included among these newer techniques are the Chinese hamster ovary (CHO) cell elongation assay (60), the CHO floating cell assay (32,48), the pigeon erythrocyte lysate assay (26), two new agar plate techniques for detecting toxin from Salmonella colonies, and an enzyme linked immunosorbent assay (32,33).

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Detailed explanations of each assay are presented later in this section. The behavior of Salmonella toxin in each of these assays is indistinguishable from that of purified cholera toxin, and we have adopted the policy of expressing Salmonella toxin values in terms of cholera toxin equivalents (ng/ml) based on direct comparison in each assay with a set of cholera toxin standard solutions. Antigenic and biologic similarities of Salmonella toxin to cholera toxin have been reported previously (55,60).
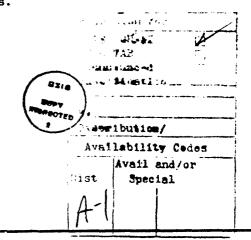
We have used these rapid techniques to determine the optimum cultural parameters for synthesis and release of the Salmonella toxin. The mitomycin C phenomenon, in which increased concentrations of Salmonella toxin appear in culture filtrates (46), has now been attributed to phage induction. We now know that toxin synthesis is widespread among Salmonella isolates, but the amount of toxin produced varies considerably from strain to strain and is substantially lower than the amount of cholera toxin produced by some laboratory strains of Vibrio cholerae. Best yields of the Salmonella toxin are achieved by culture in CYE (casamino acids-yeast extract) broth at 37°C. Shaking of flask cultures is frequently done, but our data indicate that toxin yields are comparable in still flask cultures and in anaerobic cultures. We suspect that nutritional factors affect the natural release of the toxin from growing Salmonella cells in vitro and perhaps in vivo. Cultural parameters affecting the release of toxin may explain the lack of a fluid accumulation response and the lack of an increase in tissue cyclic AMP in intestinal loops of adult rabbits challenged with certain Salmonella strains (e.g. strain SL 1027). Strain SL 1027 is now known to synthesize Salmonella toxin in CYE broth in vitro, but the amount of toxin released from the cells seems to vary in other culture media. We will examine the influence of other environmental factors on toxin release in the coming year.

We hope to establish that the documented rise in cyclic AMP in the intestinal mucosa during experimental salmonellosis is in response to the cholera toxin-like Salmonella toxin released from the bacterial cells. Data is provided in this section that supports the previous report of Gianella et al. (24) that elevation of cyclic AMP occurs in tissue from intestinal loops challenged with a fluid accumulating strain of Salmonella (SR11). Strain SL 1027 fails to elicit a fluid accumulation effect and does not elevate mucosal cyclic AMP. When Salmonella strains are grown in HMEM tissue culture medium with 2% fetal calf serum, culture filtrates of some strains (e.g., TML-R66 and W118-2) cause a rise in cyclic AMP levels of cultured Henle intestinal epithelial cells. Filtrates of other Salmonella strains (e.g., SL 1027 and M206) as well as the uninoculated culture medium do not cause a rise in cyclic AMP levels of these cultured intestinal cells. If the elevation in cyclic AMP levels in Salmonella infected intestinal loops results from the inflammatory response, as proposed by Giannella prior to discovery of Salmonella toxin (24,29), how can the rise in cyclic AMP levels in cultured Henle intestinal epithelial cells be explained, since no inflammatory cells are present? Additional data is supplied which indicates that Salmonella toxin acts directly on adenylate cyclase in the pigeon erythrocyte lysate assay. The latter system by design is unaffected by endogenous bacterial cyclic AMP. We will pursue in vivo studies to clarify the role of the Salmonella toxin as an adenylate cyclase stimulator in the pathogenesis of salmonellosis.

We have not included extensive details of our preliminary efforts toward purification of the Salmonella toxin; however, several fermenter cultures of Salmonella have been prepared. Preliminary testing of a few ion exchange methods has revealed that the Salmonella toxin binds to positively charged resins at neutral pH and can be eluted by lowering the pH to 4.0. Chromatography of the toxin preparations on Sephadex G-100 and G-150 has

produced partial purification (55,59), and isoelectric focusing of small quantities of toxin has revealed the isoelectric point to be 4.3-4.8 (32,55,59). Search for a selective, affinity procedure is seriously needed because of the low concentration of toxin in cell sonicates and culture filtrates. Preliminary studies indicate that the toxin has an affinity for chitin, that is similar to the Shigella cytotoxin (50). The latter method is inexpensive and appears to yield partial purification. Efforts to purify the Salmonella toxin will be intensified in future years now that cultural parameters in flask cultures have been carefully studied. We have purposefully delayed major efforts toward this aspect of the project until now, since very low concentrations of the Salmonella toxin were being produced. Fermenter cultures are currently being grown, using a 14 liter vessel to study the effect of some fermentation variables (e.g., aeration, stirring, temperature, and pH) on toxin yield. At present, we anticipate that highest yields of toxin will be produced when fermenter cultures are grown at 37°C with 450-500 RPM agitation. Preventing cultures from becoming acid (<7.0), and allowing the pH to rise to 8-8.5 also enhances toxin release, as reported for V. cholerae by Callahan and Richardson (7). Salmonella SL 1027 growing in CYE broth or Salmonella 8994 growing in a chemically defined medium called M-9, supplemented with biotin and selected amino acids, yields the highest levels of toxin production. Salmonella 8994, growing in the supplemented M-9 medium, retains most of the toxin inside the bacterial cells, while Salmonella SL 1027, growing in CYE broth, releases approximately 50% of its toxin into the culture medium. Selection of the appropriate time for toxin release from the cells, particularly after removal of the culture medium, may be important to minimize dilution of the toxin by large volumes of culture medium. No major problems are anticipated in large scale production of this toxin or its subsequent purification, except that toxin yields will be low. We estimate that 20 liters of fermenter culture could yield 1 mg or more of Salmonella toxin.

Finally, we have begun investigation of a "new" heat labile, cytotoxic factor found in sonicated cell preparations of several Salmonella strains grown in fermenter cultures. Cell sonicates containing the cytotoxin cause rounding and detachment of Vero cells similar to cytotoxins of other enteric bacteria (20,37). Protein synthesis in these toxin treated cells is significantly diminished within a few hours, and chromium release experiments indicate that the effect on protein synthesis is not due to cell lysis. The relationship, if any, of this cytotoxin to the pathogenesis of salmonellosis is unclear, but the toxin might cause tissue damage or aid the Salmonella in invading the intestinal epithelial cells.

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Peterson, J.W.

A. Table of Contents

B. Final Report

1. Summary of Progress

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toxin contained in culture filtrates and sonicated cell preparations. Included among
these newer techniques are the Chinese hamster ovary (CHO) cell elongation assay (60),
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2. Detailed Report

A. Growth Curve - Influence of Cultural Conditions on Mitomycin C Mediated Bacteriophage Induction and Release of Salmonella Toxin

Several isolates of Salmonella were examined for their capacity to synthesize and release a cholera toxin-like toxin that exerted a biological effect on Chinese hamster ovary (CHO) cells. The Salmonella toxin, contained in cell sonicates and culture filtrates, was expressed in cholera toxin equivalents (ng), since the CHO cell responses of the two toxins were indistinguishable. Comparative titrations of the Salmonella preparations were also performed using an enzyme-linked immunosorbent assay (ELISA) specific for cholera toxin antigen. During the growth of Salmonella strain SL 1027, the amount of Salmonella toxin synthesized was low (nanogram levels), but was detectable in cell sonicates as early as 6 hours after culture inoculation and reached maximal levels by 12 hours. Salmonella toxin antigen was undetectable in control culture filtrates until 48 hrs, but the addition of mitomycin C at 8.5 hrs resulted in a sudden appearance of toxin antigen at 10-12 hours reaching maximum at 14 hours. A large peak of CHO cell activity was observed at 48 hours in the control culture, but significant CHO cell activity was detected as early as 14 hours. A larger amount of CHO cell reactive material was observed as early as 10 hours in cultures grown with MTC. The mechanism of the MTC mediated phenomenon yielding more toxin in culture filtrates was associated with bacteriophage induction. A bacteriophage titration using a susceptible Salmonella strain revealed free bacteriophage in MTC culture filtrates (but not control filtrates) at 12 hours. Toxin production was greatest when cultures were grown at 30-37°C and lowest at 25°C. The inoculum size and degree of culture aeration (agitation) had little effect on synthesis of the toxin, and comparable toxin production occurred during anaerobic growth.

B. Nutritional Requirements for Synthesis of Salmonella Toxin.

Amino acids requirements. A chemically defined medium, M-9 salts, was selected for the nutritional study since it has been shown by Neidhardt et al. (47) to support the growth of enteric bacteria. M-9 salts contained the following ingredients: KH_2PO_4, Na_2HPO_4, NH_4Cl, $MgSO_4$, $CaCl_2$, NaCl, N-tris(hydroxymethyl)-methyl

glycine (tricine), morpholinopropane sulfonate (MOPS), and glucose. The amino acids requirement was identified by placing them into groups of four as described by Callahan and Richardson (7). Each of the 18 amino acids was used at a final concentration of 200 µg/ml, except for phenylalanine and valine (150 µg/ml); isoleucine (100 µg/ml); and tyrosine, tryptophan, and cystine (50 µg/ml).

Salmonella 8994, grown in shake flasks containing M-9 salts and different combinations of amino acids, was harvested after 18 hours of growth at 37°C. The cultures were centrifuged and supernatants were filtered through sterile Millipore filter units (0.20 µm). The cell pellets were washed once with phosphate diluent (P.D.) and resuspended in 10 ml P.D. prior to sonication. Each preparation was sonicated at 65 watts for 5 minutes in an ice-bath (4°C). After sonication, the sonicates were centrifuged and filter-sterilized. Both the filtrates and sonicates were tested for the presence of toxin by the CHO floating cell assay.

Results revealed the effect of amino acids on the growth of the culture, protein concentration, and toxin levels of both the filtrates and sonicates of Salmonella 8994 in complex as well as M-9 salts media. As expected, the complex medium CYE (Casamino acids-yeast extract) appeared superior to M-9 salts medium in the stimulation of cell growth as well as in the synthesis of toxin. Additionally, sonicates of all cultures contained most of the total protein of Salmonella cells. It was of interest to note that, with the exception of CYE and culture number 5 (M-9 salts medium containing his, gly, cys, and met), almost all the detectable toxin was found in the cell sonicates, indicating that Salmonella toxin was largely intracellular in nature when cells were grown in a simple medium. The effects of culture medium on release of Salmonella toxin may facilitate future attempts to isolate and purify this toxin. Clements and Finkelstein (10) have previously shown that most of the E. coli enterotoxin was located intracellularly, and we have noticed that the ratio of intracellular to extracellular toxin is largely dependent on the type of medium in which the Salmonella are grown.

Generally, there was no direct correlation between cell growth and toxin synthesis in cultures grown in the salts medium. Most groups of amino acid mixtures appeared to stimulate the synthesis of Salmonella toxin. The addition of all 18 amino acids to the salts medium caused approximately a six-fold increase in toxin synthesis. A more detailed examination of the effect of individual amino acids on toxin synthesis indicated that amino acids possessing the highest stimulatory effects were those that contained charged polar R groups (asp, glu, his, lys, and arg). With the exceptions of phe and leu, amino acids that consisted of nonpolar R groups were only moderately stimulatory.

Effect of different carbon sources on toxin synthesis. Glucose has often been employed as a carbon source in many nutritional studies. It was of interest to know if other carbon sources would also stimulate toxin synthesis in Salmonella. The results indicated that cells grown in the presence of glycerol contained the highest amount of both intracellular and extracellular toxin. In contrast, glucose was observed to be the least stimulatory as compared with all other carbon sources tested. In order to confirm this antagonistic effect of glucose, toxin levels in filtrates and sonicates were also tested by the ELISA assay (32,53). As expected, this antigenic assay revealed that glycerol and glucose were the highest and the least stimulatory, respectively (data not shown). In addition, sonicates of the above were heated at 100°C for 15 minutes. Results showed that toxin activity in all sonicates was virtually destroyed, indicating that the toxin is heat-labile.

Requirement for vitamins and metal cations. Since complex media for culturing bacteria contain many unknown growth factors, it was necessary to determine which vitamins were important for synthesis of Salmonella toxin. An approximate two-fold increase in toxin activity was observed in cultures supplemented with biotin, while riboflavin was only slightly stimulatory. Generally, all other vitamins were either non-stimulatory or inhibitory for toxin synthesis. Manganese was the only metal cation that stimulated toxin synthesis; iron had no effect on toxin production.

In summary, the nutritional studies indicated that a synthetic culture medium for toxin production should be comprised of M-9 salts containing glycerol, selected amino acids (phe, ser, glu, his, lys, and arg), biotin, riboflavin, and the metal cation Mn^{++}. This supplemented M-9 medium is less expensive to prepare than CYE and can yield 82% of the toxin produced by Salmonella growing in CYE. The simple medium may also facilitate purification of the toxin, since it is completely dialyzable.

C.   Intestinal Cyclic AMP Responses of Adult Rabbits to Intestinal Challenge with Live Salmonella and Salmonella Culture Filtrates.

We began the study of Salmonella mediated pathogenesis after examining the observations of Giannell, et al. (23,24). These investigators discovered that Salmonella strains causing fluid accumulation in rabbit intestinal loops also caused significant increases in intestinal tissue cyclic AMP. Salmonella SL 1027 was a genetically marked LT-2 strain which invaded rabbit ileum, but failed to elicit fluid accumulation and did not elevate intestinal cyclic AMP. We first attempted to substantiate previous data by Giannella et al. The data indicated that Salmonella SR11 elicited a fluid accumulation response in each of the four rabbits in a manner comparable to TML. Salmonella SL 1027 did not elicit fluid accumulation, which supported earlier reports (24). In 3 of 4 rabbits, the fluid accumulation response in the loops to Salmonella SR11 was accompanied by an approximate 10-fold increase in mucosal tissue cyclic AMP compared to the 2-fold rise reported earlier for strain TML (24). The tissue from rabbit number two may have been incorrectly taken since it failed to show any rise in cyclic AMP. Our data, therefore, support the previous observation made by Giannella et al. (24) regarding elevation of cyclic AMP, and we plan additional in vivo experiments as described in this proposal to elucidate the mechanism of cyclic AMP elevation and its relationship to the pathogenesis of salmonellosis.

Recently, we prepared 30x concentrates of Salmonella CYE culture filtrates by dialysis of the filtrates against carbowax. When 2 ml volumes were injected into the intestinal lumen of adult rabbit loops, no fluid accumulation was observed. These negative findings have continued to be discouraging, but reflect the low ng/ml concentration of Salmonella toxin in the filtrates. We have observed positive fluid responses with partially purified, concentrated preparations of delayed PF. These results have not yet been accepted for publication because of a reviewer's criticism that a dose response curve was not included; however, only limited amounts of Salmonella toxin were available at that time for those studies. We are certain that the data reported are valid, since loop activity could be blocked by preincubation with monospecific cholera antitoxin. We will strive to substantiate and publish these data later.

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D. Stimulation of Adenylate Cyclase and Elevation of Cyclic AMP Levels by _Salmonella_ Filtrates

1. Elevation of cyclic AMP levels in intact Henle intestinal epithelial cells.

Our observation that _Salmonella_ toxin caused elongation of Chinese hamster ovary (CHO) cells suggested to us that this toxin, like cholera toxin, caused an increase in cyclic AMP levels. To determine if a comparable rise in cyclic AMP occurred in intestinal epithelial cells in the absence of an inflammatory cell response, cultured Henle intestinal epithelial cells were exposed to _Salmonella_ toxin and their cyclic AMP levels were measured. Several strains of _Salmonella_ were grown in HMEM containing 2% fetal calf serum. After removal of the bacterial cells by centrifugation, the cultures were brought to neutral pH and filter sterilized. Monolayers of Henle intestinal epithelial cells were then incubated in these filtrates at 37°C. After 4 hours of incubation, the monolayers were washed twice in PBS and collected for cAMP and protein determinations as described by Guerrant (30). Filtrates of strains TML and W118-2 increased cAMP levels in the cells, while filtrates of strains M206 and SL 1027 did not. Giannella et al., reported that strains TML, W118-2, and M206 caused fluid accumulation in rabbit intestinal loops, while strain SL 1027 invaded the intestinal epithelium, but did not cause fluid accumulation (23). Thus, this experiment showed some correlation between the ability of _Salmonella_ strains to induce fluid accumulation in vivo and their capacity to increase cAMP levels in eukaryotic cells cultured in vitro. The serum enriched medium used in this experiment for cultivation of the _Salmonella_ may have influenced toxin release by these isolates. Serum factors could also be important determinants of toxin release in vivo.

2. Stimulation of adenylate cyclase in pigeon erythrocyte lysates.

To demonstrate that the increase in cyclic AMP levels was due to activation of adenylate cyclase, we tested _Salmonella_ filtrates in the pigeon erythrocyte lysate assay (26,27). As described in the previous section, the _Salmonella_ strains were grown in HMEM containing 2% fetal calf serum. In this medium, the bacteria released increased amounts of toxin even in the absence of mitomycin C. After 24 hours of growth, the cultures were centrifuged and filter sterilized. Samples of the filtrates were then tested in the pigeon erythrocyte lysate assay. The results of two separate experiments showed that adenylate cyclase was stimulated by most of the _Salmonella_ filtrates. Furthermore, the adenylate cyclase activating activity was heat labile.

Thus, we concluded from these experiments that _Salmonella_ toxin does increase cyclic AMP levels in eukaryotic cells. As in the case of cholera toxin, the increased cyclic AMP levels caused by _Salmonella_ toxin are mediated by an increase in adenylate cyclase activity. Since the experiments were performed in vitro, the rise in cyclic AMP levels occurred in the absence of inflammatory cells.

E. _Salmonella_ Cytotoxic Factor

Recently, during the initial stage of purifying the heat-labile _Salmonella_ toxin, another potential virulence factor was detected in chromatography fractions of crude fermenter concentrates of _Salmonella_ strain 9630. This factor appeared to be cytotoxic, causing rounding or cell death to CHO cells. The cytotoxin

was further tested with Vero cells, a cell line commonly used for the detection of E. coli cytotoxin (37), and found to destroy the cell monolayer. Moreover, this cytotoxic factor could be inactivated by boiling for 30 minutes.

A modified cytotoxicity (cell-detachment) test according to Gentry and Dalrymple (20) was employed to monitor and quantitate the Salmonella cytotoxin present in both culture filtrates and cell sonicates. A survey of several strains of Salmonella has indicated that most of the cytotoxin is intracellular in nature. Dose-response and heat-lability studies were performed with cytotoxin from cell sonicates of five Salmonella strains. Crude sonicates containing the cytotoxin were inactivated by boiling.

The molecular mechanism by which shiga toxin causes cytotoxicity to eukaryotic cells involves inhibition of protein synthesis (6). Preliminary data obtained in our laboratory on Salmonella cytotoxin appears to reveal a similar pattern. The amount of ^{3}H-leucine incorporated into protein was significantly less when Vero cells were cultured in the presence of crude cytotoxin. The diminished uptake of ^{3}H-leucine, however, could have resulted either from a direct effect on protein synthesis machinery or from an effect on cell membrane integrity. To investigate the latter possibility two kinetic experiments were performed. In the first experiment, confluent monolayers of Vero cells were incubated with crude Salmonella cytotoxin, and the effect on protein synthesis was measured after 0.5 hour intervals for up to 4 hours. Inhibition of ^{3}H-leucine incorporation into protein could be observed as early as 1.5 - 2.0 hours and thereafter the inhibitory effect of the cytotoxin continued with increased incubation. Concomitantly, the effect of crude Salmonella cytotoxin on ^{51}Cr-release from Vero cells was examined using the same time intervals. Prior to the treatment with cytotoxin, confluent monolayers of Vero cells were incubated with 100 µCi/ml of ^{51}Cr for 4 hours. The ^{51}Cr-treated cells were then incubated with the cytotoxin, and the reaction was terminated after 4 hours. The results indicated that there was no appreciable damage to the Vero cell membranes as shown by the negligible values for percent cell lysis although protein synthesis was significantly inhibited.

F. Development of Plate Assays for Future Genetic Investigations

1. Blood Agar Plate Assay for Detection of Toxin Released from Salmonella Colonies

One major difficulty in studying the genetics of toxin production by Salmonella was the lack of a quick, inexpensive, and relatively easy assay for testing many isolates for toxin production. For this purpose, we have recently developed a modification of the radial, passive immune hemolysis assay of Bramucci and Holmes (4). Syncase agar plates are overlaid with 5 ml of molten syncase-blood agar containing 50-100 bacteria. After 24 hours incubation at 37°C, the plates are overlaid with 3 ml of molten syncase agar containing 100 g of polymyxin B and 1 mg of lysozyme. The plates are then incubated for another 24 hours at 37°C. Treatment of the Salmonella colonies with polymyxin B and lysozyme causes them to release detectable levels of toxin. The toxin diffuses through the agar and binds to ganglioside constituents on the surface of erythrocytes in the agar. The plates are then overlaid with soft agar containing guinea pig complement and specifically purified antibody to cholera toxin. When the antitoxin and complement react with the toxin that has attached to the erythrocytes, the erythrocytes lyse, causing a zone of hemolysis around toxin-producing colonies. This plate assay may be an extremely valuable tool in screening mutants and recombinants for toxin production.

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Peterson, J.W.

2. Autoradiograph Plate Assay

Our laboratory has developed a second plate assay that uses cyanogen bromide (CNBr)-activated filter paper and specifically purified cholera antitoxin. Colonies of Vibrio cholerae, Escherichia coli, or Salmonella species are grown on CYE agar plates for 18-24 hours. The plates are inverted over a filter paper disc saturated with chloroform to enhance release of intracellular contents by lysis of the bacterial cells. Subsequently, a top agar layer, containing SDS and lysozyme, is applied to each plate. Plates are also spotted with 10 µl each of dilutions of cholera toxin to measure assay sensitivity. Whatman No. 1 filter paper discs, previously sensitized with specifically purified cholera antitoxin and stored at -20°C, are then laid on the surface of the agar overlay. After 9-10 hours of additional incubation at room temperature, the sensitized paper discs are removed and washed in a Buchner funnel with PBS. Each is then floated in 10 ml of ^{125}I labeled, specifically purified cholera antitoxin for 5 hours at room temperature. Following extensive washing again in PBS, the discs are dried and taped to X-ray film for 1-2 days. The black spots on the developed autoradiograph reveal the location of cholera toxin antigen surrounding colonies of toxinogenic strains. Positive autoradiographic data is currently being obtained with strains of Salmonella, Escherichia, and Vibrio. The assay can detect little as 1 ng of purified cholera toxin. We are currently assessing the feasibility of this assay for detection of toxin production from a variety of clinical isolates of Salmonella and E. coli, but will delay the presentation of detailed observations until more experience is acquired. Like the other plate assay for toxin production, this assay appears to have considerable potential value.

G. Bacteriophage Investigations

Several strains of Salmonella were screened for the presence of temperate bacteriophages. The strains, growing in CYE broth, were subjected to mitomycin C induction (0.5 µg/ml). Ten-fold dilutions of culture filtrates from the induced strains were dropped onto CYE agar plates containing bacterial indicator lawns of the same strains. Based on preliminary data, there did not appear to be a correlation between toxin production and phage association, when MTC was used as the inducing agent. An identical experiment utilizing U.V. light as an inducer will, be conducted and the results should indicate whether it is an efficient inducer compared to MTC.

We have now completed our bacteriophage experiments and have concluded that temperate phage do not carry the genes for Salmonella toxin synthesis. Experiments were performed that involved inducing bacteriophage from lysogenic strains of Salmonella and the construction of new lysogens by infecting phage-free Salmonella recipients. Bacteriophage were MTC induced from donor Salmonella strains TML-R66, M206, SR11, SL 1027, and 986. Salmonella strains 2000 and Q1 were used as phage-free recipient lawns onto which drops of a cell-free phage preparation of each donor we placed. Portions of the area of lysis were harvested with a sterile wire loop and streaked onto CYE agar plates. Two CYE agar plates, one containing a recipient strain of Salmonella in a soft agar overlay, were placed on grid templates. Twenty-four selected isolated colonies on the CYE agar streak plates were inoculated onto each of the two grid plates with sterile toothpicks. The plates were incubated at 37°C for 24 hours prior to examination. Clearing around the colonies on the recipient lawn indicated phage lysis, and therefore, the formation of a new lysogen. The duplicate cultures grown on the plain CYE agar plate were used as the donor for repeat

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experiments. Five newly constructed lysogens were grown in CYE broth for 24 hours at 37°C and the filtrates and sonicates of each lysogen were assayed by the ELISA and CHO floating cell assay. Salmonella toxin values for the newly constructed lysogens were not significantly different from those of the original donor and recipient strains, indicating that the bacteriophage associated with Salmonella strains utilized in this study did not provide the genetic information for toxin synthesis.

H.   Miscellaneous Observations

1.   Suckling mouse assay – The suckling mouse assay (16) was set up in our laboratory to determine if crude filtrates containing Salmonella toxin elicit a positive fluid response in this model. Although positive fluid responses were observed with filtrates from a strain of E. coli known to elaborate ST, no fluid accumulation occurred in mice fed filtrates from several Salmonella strains. We concluded that the Salmonella toxin was negative in this assay or that it was insufficient in concentration to elicit a positive response.

2.   Adrenal Cell Assay – A collaborative arrangement was established with Dr. Sam Donta at the University of Iowa to measure toxin in 10 filtrate preparations. Salmonella culture filtrates were assayed by the CHO floating cell assay, as well as the ELISA, prior to refrigerated shipment to Iowa. Dr. Donta assayed the 10 coded samples using the adrenal cell assay (17). He then assigned them a second code, added five additional samples, and returned them to us for reassay. We again assayed the samples as a blind study and compared these results with the initial titrations. No positive data with Salmonella filtrates was obtained by Dr. Donta using the adrenal cell assay. In contrast, the CHO cell assay and ELISA correlated well in estimates of Salmonella toxin content. Furthermore, the blind assay correlated quite well with the initial titrations. We could not explain the lack of positive results in the adrenal cell assay. No further studies are planned, but the lack of adrenal cell responses may reflect an inherent difference between Salmonella toxin and cholera toxin, or it may simply depict a difference in assay sensitivity.

3.   Mouse LD_{50} Determinations – A preliminary study was performed to determine if a correlation existed between the capacity of Salmonella strains to produce the heat labile toxin and lethal infection of mice. Adult Swiss Webster mice were distributed into groups of 8 mice. Doses of viable cells of each Salmonella strain, ranging from 10^0–10^5, were injected by the intraperitoneal route without suspension in mucin. The mice were examined daily and deaths were recorded for 21 days following injection. Of the strains tested, the data showed that only SR11 and 986 were highly virulent in this model, exhibiting LD_{50} values of less than 1000 cells, while most strains were weakly virulent (LD_{50} >10^5).

Although the lack of correlation between LD_{50} and toxin producing capacity suggested that the Salmonella toxin plays little role in systemic infection leading to animal death, an additional experiment was designed to examine further its possible involvement. Adult, Swiss Webster mice were distributed into three groups: Nonimmunized controls, immunized with 1 µg cholera toxin, or immunized with 10 µg cholera toxin. The cholera toxin immunized mice received the toxin dose in 0.5 ml of Tris buffer by the intraperitoneal route two weeks prior to live cell challenge. All mice were challenged with varying doses of live Salmonella SR 11 by the intraperitoneal route. The data indicated that immunization with cholera toxin did not protect mice against live cell Salmonella challenge.

I. Current Personnel - Salmonellosis Program

    1. Postdoctoral fellow

        Felix C.W. Koo, Ph.D.

    2. Graduate students - predoctoral fellow

        N. Christine Molina, B.A.

    3. Graduate students - master's degree candidate

        Ingrid Duebbert, B.A.

    4. Technical personnel

        Technical personnel associated with this program could not be supported beyond 7/31/81 because of a delay in funding of this proposed research.

J. Publications Relating to Salmonellosis Research

    1. Articles in journals.

        Sandefur, P.D. and J.W. Peterson. 1976. Skin permeability factors culture filtrates of Salmonella species. In Proc. Twelfth Joint Conference on Cholera (1976), The United States-Japan Cooperative Medical Science Program. p. 185-199.

        Sandefur, P.D. and J.W. Peterson. 1976. Isolation of skin permeability factors from culture filtrates of Salmonella typhimurium. Infect. & Immun., 14:671-679.

        Sandefur, P.D. and J.W. Peterson. 1977. Neutralization of Salmonella toxin induced elongation of Chinese hamster ovary cells by cholera antitoxin. Infect. & Immun., 15(3):988-992.

        Peterson, J.W. and P.D. Sandefur. 1979. Evidence of a role for permeability factors in the pathogenesis of salmonellosis. Amer. J. Clin. Nutrition., 32:197-209.

        Molina, N.C. and J.W. Peterson. 1980. A cholera toxin-like toxin released by Salmonella species in the presence of mitomycin C. Infect. Immun. 30:224-230.

        Peterson, J.W. 1980. Salmonella toxin. Pharmac. Ther. 11:719-724.

        Peterson, J.W., C.W. Houston, and F.C.W. Koo. 1981. Influence of cultural conditions on mitomycin C- mediated bacteriophage induction and release of Salmonella toxin. 32:232-242.

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Houston, C.W., F.C.W. Koo, and J.W. Peterson. 1981. Characterization of *Salmonella* toxin released by mitomycin C treated cells. Infect. Immun. 32:916-926.

2. In Press.

Koo, F.C.W. and J.W. Peterson. 1981. The influence of nutritional factors on synthesis of *Salmonella* toxin. J. Food Science (In Press).

Houston, C.W., C.P. Davis, and J.W. Peterson. 1981. *Salmonella* toxin synthesis is unrelated to the presence of temperate bacteriophages. Infect. Immun. (In Press).

3. In preparation.

Peterson, J.W., P.A. Dunn, F.B. Martin, and J.P. Craig. 1981. Enterotoxic activity of *Salmonella* delayed permeability factor. Infect. Immun. (Submitted Feb. 1980 - returned for additional research and revisions).

Koo, F.C.W. and J.W. Peterson. 1981. The role of a cytotoxic factor in the pathogenesis of salmonellosis.

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